Sirtuins in Kidney Injury and Disease

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Acute kidney injury (AKI) is an extremely common medical affliction resulting from hypoxic, nephrotoxic and septic insults affecting approximately 20% of all hospital patients and up to 50% of patients in the intensive care unit. There are currently no therapeutics for patients that suffer AKI. Much recent work has focused on designing and implementing therapeutics for AKI. The sirtuins are a family of enzymes that play critical roles in regulating many cellular and biological functions. There are 7 mammalian sirtuins (Sirt1-7) that are known to regulate the acylation of a wide variety of pathways. Furthermore, all but one of the mammalian sirtuins have been shown to be important in mediating AKI based on preclinical studies. These diverse enzymes show exciting potential for therapeutic manipulation. While considerable research has been done on SIRT1 and its role in the kidney, much less is known about mitochondrial SIRT5 in the context of AKI. My research has strove to elucidate the role of SIRT5 in acute kidney injury through *in vivo* mouse studies and *in vitro* means with a particular focus on its function as a regulator of fatty acid oxidation.

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Preface

I would like to thank the University of Pittsburgh and my mentor, Sunder Sims-Lucas, for their guidance and support during this process. I also wish to thank my friends and family for their unwavering support.

1.0 Background and Significance

1.1 Acute Kidney Injury

Acute kidney injury (AKI) is a significant health care concern associated with high morbidity and mortality [1, 2]. Approximately 20% of hospitalized patients have AKI and 20- 60% of adult critically ill patients have AKI [3]. AKI is characterized by an abrupt decline of renal function, resulting in an inability to maintain electrolyte, acid-base, and fluid homeostasis [4]. AKI is a complex and multifactorial disease typically occurring as a mixed etiology of ischemia, nephrotoxicity, and sepsis. Many groups are working to identify the underlying mechanisms involved in AKI, including apoptosis, dysregulation of metabolism, autophagy, inflammation and the cell-cycle [5-9]. However, despite considerable improvements to our understanding of the pathophysiology of AKI, the exact mechanisms are still poorly understood, and no specific therapy exists.

1.2 Pathogenesis of AKI

Acute kidney injury is a complex and multifactorial disease. Although the exact pathogenesis of AKI depends on the cause of injury, the most common causes of AKI in patients is a mixed etiology of ischemia, nephrotoxicity, and sepsis. Despite potential differences in the initial injury, subsequent injury responses involve similar pathways and cell types. Among the molecular and cellular responses to injury are cell death of the tubular epithelial and endothelial

cells, innate and complement-mediated immune system activation, and impaired cellular metabolism.

Models of ischemic and toxic injury to the kidneys shows a special susceptibility to injury of the renal tubular epithelial cells (RTEC). In all the complexity of AKI, it is generally accepted that the underlying basis of kidney injury is impaired energetics in the highly metabolically active nephron segments. The proximal tubular epithelial cells are especially sensitive to injury because they require more active transport mechanisms than other renal cell types. As such, they are heavily invested with mitochondria and peroxisomes to meet energy demands. When an injury or stress exceeds the mitochondria's ability to sense and respond to changes in nutrient availability, apoptosis and necrosis are commonly initiated [10]. Several key enzymes and pathways are responsible for the efficient metabolism of resources in the kidney parenchyma. On the mitochondrial side, carnitine palmitoyletransferases (CptI and CptII) are critical enzymes essential for fatty acid oxidation. Apoptosis can activate intrinsically when the mitochondria fragments in response to decreased energy supply or when cytochrome c is released following outer mitochondrial membrane permeabilization [11]. Apoptosis of the tubular epithelial cells is further mediated by a network of factors including tumor suppressor protein p53, B-cell lymphoma 2 (BCL2) family proteins, and caspases [12]. The consequences of such a tubular injury are many-fold. Beyond the intrinsic cell death, various cytokines and chemokines are released from the damaged cells, triggering further cell death in adjacent cells and a robust immune response. Moreover, since the nephron's natural function is to filter and reabsorb many substances from the tubular lumen, damage can result in toxic accumulation of these substances for surrounding epithelial cells, initiating a cascade of downstream molecular and cellular effects.

The endothelial cells of the renal microvasculature are tightly integrated with the nephron and play a critical role in the pathogenesis of AKI. Several studies have provided evidence supporting cross-talk between the endothelial cells and RTEC [13]. Unlike renal tubular epithelial cells, endothelial cells lack the capacity for regeneration following severe injury. Such injuries can interfere with normal vascular activity, influence coagulation cascades, and trigger inflammatory responses [14]. This reduction in blood flow and impaired metabolism can have injurious consequences for the interdependent tubular epithelial cells.

An inflammatory response follows initial tubular and endothelial injury and amplifies injury to the RTEC and endothelial cells. Damaged endothelial cells upregulate a variety of adhesion molecules with leukocyte counterreptors that initiate an inflammatory cascade of cytokines, chemokines, and reaction oxygen species (ROS) when activated [15]. Inflammatory mediators are also released by the RTEC. RTEC produce interleukin (IL)-1, IL-6, IL-8, and transforming growth factor beta (TGF-B) while leukocytes produce IL-1, IL-8, ROS, and eicosanoids [16]. Both the innate and adaptive immune systems are involved in the pathogenesis of AKI and virtually every immune cell has been implicated in AKI [17-20]. Briefly, the early immune response consists of activation of dendritic cells and macrophages that produce cytokines and chemokines leading to an influx of leukocytes [21]. Dendritic cells activate cluster of differentiation 4 (CD4)+ natural killer T cells and promote inflammation [19]. Following initial injury, regulatory T cells and macrophages suppress ischemic injury or initiate repair of the injured tissue [22, 23]. The complement system is also prominent in several types of AKI and together with toll-like receptors (TLRs) acts as an early sensor of tissue injury. Blockade of complement activation prevents many of the downstream inflammatory manifestation of AKI [24].

Peroxisome proliferator-activated receptors (PPARs) are important nuclear receptor proteins that regulate diverse cellular functions such as cell survival, lipid metabolism, and inflammatory response. All three types of PPARs (PPAR-α, PPAR-γ, and PPAR-β/δ) have shown protective roles in animal models of AKI [25-28] For example, PPAR-α-deficient mice are more susceptible to sepsis-associated AKI and have worse kidney function likely due to reduced fatty acid oxidation and increased inflammation [26]. Additional mechanisms of protection have been elucidated for PPAR-α in the context of kidney injury. Ischemic-AKI and cisplatin-AKI decrease expression and activity of multiple enzymes involved in fatty acid oxidation and glucose metabolism. Proximal tubule-specific overexpression of PPAR-α is sufficient to prevent loss of these enzymes and ameliorate kidney injury [29].

In summary, tubular cell death is a central feature of AKI and the pathogenesis of AKI is a complex interrelationship between the epithelial, endothelial, and immune cells. Following initial injury, these cells act in concert to promote inflammation in a positive feedback loop to exacerbate kidney injury.

1.3 Sirtuins

Sirtuins are a multi-dimensional group of genes that may provide insight into protective mechanisms during AKI. Sirtuins are a family of nucleotide adenine dinucleotide (NAD+)-dependent class III histone deacylases. The first identified sirtuin, Sir2, was found in budding yeast *Saccharomyces cerevisiae* and described as a regulator of transcriptional silencing of mating-type loci [30]. The discovery of Sir2's dependence on NAD+ revealed a role for sirtuins as both energy sensors and as transcriptional effectors regulating the acetylation state of histones [31].

Subsequently, many homologs of Sir2 have been discovered across all domains of life, establishing a highly conserved class of enzymes [32-34].

In mammals, there are seven sirtuins, SIRT1-7, which function to regulate metabolism and other diverse physiologic processes through direct enzymatic action on target proteins. Sirtuins act in different cellular compartments and exhibit broad enzymatic activity as deacetylases, mono-ADP ribosyltransferases, demalonylases, deglutarylases, and desuccinylases [35-37]. Apart from the classic role as histone deacetylases, a diverse set of protein targets have also been identified in the cytoplasm and mitochondria [38]. Although sirtuins have notably been studied for their role in caloric restriction and the prevention of aging-related diseases such as cardiovascular disease and diabetes, their diverse substrates and role as sensors of cellular energy balance makes them a critical player to restoring cellular homeostasis following injury [39]. The kidney is one organ that is highly susceptible to age-related diseases, and sirtuins have been implicated in the pathophysiology of chronic and acute kidney diseases [40, 41].

1.4 Sirtuins in AKI

1.4.1 Nuclear Sirtuins

There are 3 nuclear sirtuins (SIRT1, 6 and 7) that have been shown to play key roles in AKI and these are discussed below. SIRT1 is the most studied sirtuin and is localized to the nucleus (Table 1). It was originally described as a histone deacetylase, but it was soon discovered that SIRT1 deacetylates other proteins [36, 42, 43]. Following DNA damage, SIRT1 deacetylates and represses p53 to reduce cell apoptosis and senescence [44, 45]. Similarly, SIRT1 regulates the

acetylation of the forkhead box type O (FOXO) transcription factors to attenuate FOXO-induced apoptosis and cell-cycle arrest [46]. SIRT1 also regulates both members of the peroxisome proliferator-activated receptor gamma coactivator 1-alpha/estrogen- related receptor alpha (PGC-1α/ERR-α) complex, essential metabolic transcription factors which control mitochondrial biogenesis and gluconeogenesis (Table 1) [47-49]. Apoptosis and mitochondrial dysfunction play a critical role in the pathogenesis of AKI. When an injury or stress exceeds the mitochondria's ability to sense and respond to changes in nutrient availability, apoptosis is commonly initiated [10]. Apoptosis can activate intrinsically when the mitochondria fragments in response to decreased energy supply or when cytochrome c is released following outer mitochondrial membrane permeabilization [11]. Apoptosis of the tubular epithelial cells is further mediated by a network of factors including tumor suppressor protein p53, BCL2 family proteins, and caspases [12]. Given SIRT1's strong role in regulating apoptosis, it is not surprising there is a wealth of literature describing the renoprotective effects of SIRT1. In its renoprotective capacity SIRT1 was shown to protect against oxidative stress-induced apoptosis via deacetylation of FOXO3 in proximal tubular epithelial cells [50]. Further to this, in proximal tubular-specific SIRT1-overexpressing mice, cisplatin-induced injury was attenuated by maintaining peroxisome number with concomitant upregulation of catalase and reduction of renal oxidative stress [51]. In another study, it was shown that SIRT1 activates PGC-1α, resulting in renoprotection by activating mitochondrial biogenesis and improved respiration via oxidative phosphorylation [52]. Finally, SIRT1 also showed a protective effect by regulating apoptosis through deacetylating p53 and inhibiting p53-dependent transcription during cellular stress [53]. Thus, the mechanism of Sirt1 protection is mediated by suppression of apoptosis likely downstream of metabolic signaling pathways.

SIRT6 is another sirtuin that localizes to the nucleus. It functions as both a deacetylase and ADP ribosyltransferase [54]. SIRT6 plays a significant role in genomic DNA stability and repair. Sirt6 knock-out mice present with severe progeria and typically only live for 3 months [55]. SIRT6 deacetylates histone H3 at various lysine sites to maintain genome integrity and telomere function [56]. In response to DNA damage, it has been shown that SIRT6 promotes DNA end resection via deacetylation of C-terminal binding protein interacting protein (CtIP) [57]. In the context of the kidney, SIRT6 appears to be important for podocyte function and maintenance of glomerular function, as Sirt6 deletion in mice induces podocyte injury and decreased slit diaphragm protein expression [58]. Sirt6 overexpression in HK-2 cells (immortalized proximal tubular kidney cells) inhibits apoptosis induced by lipopolysaccharide and promotes autophagy while SIRT6 silencing promoted the secretion of cytokines tumor necrosis factor α (TNF-α) and interleukin-6 (IL-6) [59]. SIRT6 also deacetylates histone 3 to effectively inhibit ERK1/2 expression and reduce inflammation and apoptosis caused by cisplatin [60].

SIRT7 is a deacetylase uniquely localized to the nucleolus. Compared to other nuclear sirtuins, only a limited number of substrates have been identified. SIRT7 activates RNA polymerase I (RNA Pol I) by deacetylating upstream binding factor (UBF) [61]. Others have shown that SIRT7 regulates RNA Pol I transcription by deacetylating PAD53, a component of RNA Pol I [62]. Interestingly, lack of SIRT7 in mice shows protection against cisplatin-induced AKI. By regulating the nuclear expression of transcription factor nuclear factor kappa B (NF- κB), SIRT7 deficiency ameliorates cisplatin-induced AKI [63]. Regulation/dysregulation of NF-κB would have profound effects on the kidney response to AKI due to the role of the immune response during injury. Both the innate and adaptive immune systems are involved in the pathogenesis of AKI and virtually every immune cell has been implicated in AKI [17-20]. The protective

phenomena seen with SIRT7 deficient mice is perhaps due to SIRT7 deficiency reducing the expression of TNF-α, which normally enhances ROS production through the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex [63].

1.4.2 Mitochondrial Sirtuins

There are 3 known mitochondrial sirtuins (SIRT3, 4 and 5). While SIRT3 is responsible for global protein deacetylation in mitochondria, SIRT4 exists as a mitochondrial ADP-ribosyltransferase and SIRT5 exhibit enzymatic activities as a deacetylase, desuccinylase, and demalonylase. The roles of SIRT3 and SIRT5 have been elucidated in kidney injury and are discussed below, while the role of SIRT4 is currently unknown in this context.

As the major mitochondrial deacetylase, SIRT3 is responsible for the regulation of several metabolic enzymes and components of oxidative phosphorylation. SIRT3 deacetylates and activates mitochondrial acetyl-CoA synthetase (AceCS2), an enzyme involved in acetate utilization [64]. Similarly, SIRT3 has been shown to deacetylate long-chain acyl-CoA dehydrogenase (LCAD) to regulate fatty acid oxidation [65]. SIRT3 has also been demonstrated to deacetylate mitochondrial ribosome subunit MRPL10 to inhibit mitochondrial protein synthesis [66]. In addition, SIRT3 regulates various components of the electron chain, such as Complex II and ATP synthase, to enhance ATP levels [67, 68]. These studies reflect only part of SIRT3's wide range of functions in mitochondrial ATP production, fatty acid oxidation, mitochondrial homeostasis, and ROS management. SIRT3 plays a significant role in the kidney, especially in the proximal and distal tubular compartments which contain abundant mitochondria. Several studies have described a renoprotective role for SIRT3 due to its role in maintaining mitochondrial dynamics and energy homeostasis. Indeed, Sirt3 knockout mice administered cisplatin have more

severe AKI and compromised mitochondrial dynamics [69]. SIRT3 overexpression in mice has the effect of promoting autophagy through regulation of the mechanistic target of rapamycinAMPK/mTOR pathway and protecting against a model of sepsis-AKI [70]. SIRT3 overexpression promotes autophagy, upregulates p-AMPK and downregulates p-mTOR in cecal ligation and puncture mice, attenuating sepsis-induced AKI, tubular cell apoptosis, and inflammatory cytokine accumulation in the kidneys [71]. The blockage of autophagy induction largely abolished the protective effect of SIRT3 in sepsis- induced AKI. These findings indicate that SIRT3 protects against sepsis-induced AKI by inducing autophagy through regulation of the AMPK/mTOR pathway. SIRT3 also regulates FAO in mice by deacetylating liver kinase B1 and activating AMPK with the effect of reducing reactive oxygen species (ROS) and lipid peroxidation [72].

Most pertinent to this thesis is the mitochondrial SIRT5 was initially identified as a deacetylase targeting carbamoyl phosphate synthetase (CPS1) to regulate the urea cycle in liver [73]. However, it has since been discovered that SIRT5 functions as a demalonylase, deglutarylase, and desuccinylase rather than as a deacetylase [74]. SIRT5 has been shown to bind cardiolipin in the inner mitochondrial membrane and desuccinylate electron transport enzymes Complex I, Complex II, and ATP synthase [75]. SIRT5 desuccinylation targets identified via large-scale profiling studies suggest SIRT5 has a significant role in energy metabolism [76]. As a mitochondrial sirtuin, SIRT5 was expected to have a significant role in the kidney and in the maintenance of the kidney's energy metabolism. However, few studies have investigated the role of SIRT5 in the kidney. The major focus of my research and this thesis comprises my research into the role of SIRT5 in kidney injury and disease. This research resulted in a publication for which I

was co-first author. The results will be elaborated in the "Results and Discussion" section of this thesis.

1.5 Elucidating the Role of SIRT5 in Acute Kidney Injury

Proximal tubule epithelial cells (PTEC) are highly sensitive to damage following AKI largely due to its high metabolic rate [77, 78]. To meet energy demands, PTEC rely on fatty acid β-oxidation (FAO). Parallel FAO pathways exist in mitochondria and in peroxisomes, both of which are notably abundant in PTEC. Mitochondrial FAO directly links to the mitochondrial electron transport chain and is an oxygen-intensive source of energy. Concomitant with oxygen utilization is the formation of reactive oxygen species (ROS) which are thought to be particularly damaging during ischemic- and cisplatin-induced AKI [79, 80]. Limiting mitochondrial FAO limits cardiac injury in the heart [81, 82] and in the kidney [25].

In contrast to mitochondria, little is known about the role of peroxisomes in the kidney, particularly in the context of AKI. Peroxisomes cannot produce energy. Rather, they metabolize long-chain fatty acids to acetate and other short-chain products, which, owing to their hydrophilicity, can cross membranes to either leave the cell or enter mitochondria where they may be oxidized to completion. Peroxisomes have been observed to physically interact with mitochondria and ablation of peroxisomal function has secondary effects on mitochondrial function [83]. Peroxisomes may serve as a sink for mitochondrially-produced ROS due to the abundance of catalase and other ROS-neutralizing enzymes in the peroxisome [84, 85]. Peroxisomes may also serve to protect PTEC from the accumulation of toxic long-chain fatty acids [86].

Emerging evidence shows both FAO pathways are regulated by reversible post-translational modifications (PTM), in particular by lysine acylation and the sirtuin deacylase enzymes that remove these PTM [65, 87]. Sirt5, which is unique among the sirtuins in its substrate preference for succinyllysine, malonyllysine, and glutaryllysine [74, 88], also promotes mitochondrial FAO in liver and heart [76, 87]. Intriguingly, Sirt5 was recently shown to localize to peroxisomes. In direct contrast to its effect on mitochondrial FAO, Sirt5 was observed to suppress peroxisomal FAO in vitro and in rodent liver [89] The role of Sirt5 in the kidney has not yet been studied. Thus, we aimed to investigate the precise role of SIRT5 in AKI.

Table 1: Sirtuin expression, enzymatic activity, targets, and function

Sirtuin	Localization	Enzymatic Activity	Targets	Function	Citation
Nuclear Sirtuins					
SIRT1	Nucleus	Deacetylation	p53 FOXO3 PGC-1α	Maintenance of peroxisomes Mitochondrial biogenesis Mitochondrial homeostasis Regulation of apoptosis	[33-36]
SIRT6	Nucleus, Cytoplasm	Deacetylation, ADP-ribosylation	ERK1/2 TNF-α	Maintenance of glomerular function Podocyte function Apoptosis Autophagy	[41-43]
SIRT7	Nucleolus	Deacetylation	NF-κB	Inflammation	[46-50]
Mitochondrial Sirtuins					
SIRT3	Mitochondria	Deacetylation	LKB1 AMPK/mTOR	Mitochondrial dynamics Autophagy Oxidative stress	[56-59]
SIRT5	Mitochondria	Demalonylation, Deglutarylation, Desuccinylation	B-oxidation	Fatty acid oxidation Energy metabolism	[64]
Cytoplasmic Sirtuins					
SIRT2	Nucleolus	Deacetylation, Demyristolyation	MAPK-1	Inflammation Apoptosis	[71-72]

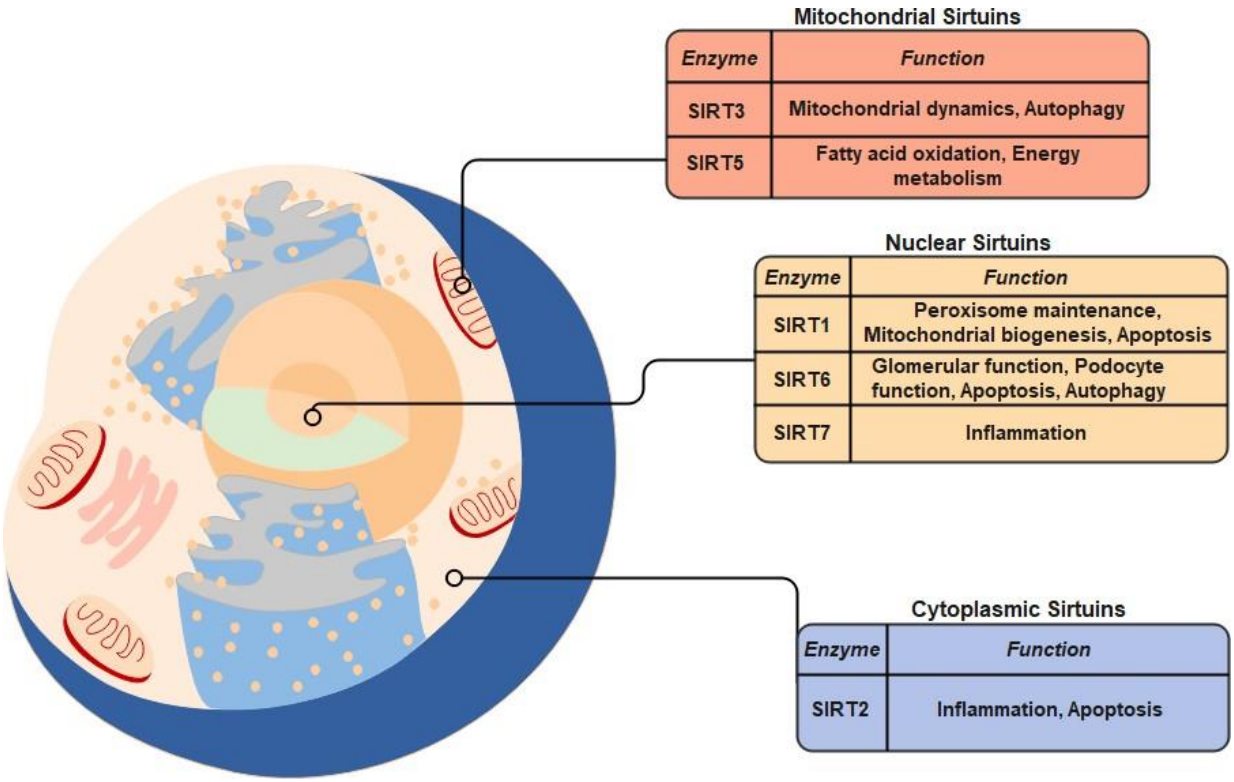


Figure 1: Sirtuins regulates different cellular and biologcal functions to impact kidney injury. SIRT3 and SIRT5 are mitochondrial sirtuins. SIRT3 maintains miotchondrial dynamics and energy homeostasis and promotes autophagy. SIRT5 is involved in fatty acid oxidation and energy metabolism. SIRT1, SIRT6, and SIRT7 are nuclear sirtuins. SIRT1 has a strong role in maintaining peroxisomes and mitochondria as well as in regulating apoptosis. SIRT6 is important for the maintenance of podocyte and glomerular function. SIRT7 is involved in inflammation through regulation of NF-κB. SIRT2 is a cytoplasmic sirtuin and regulates both inflammation and apoptosis

2.0 Methods

2.1 Ischemic- and cisplatin-AKI models in mice

Homozygous global Sirtuin5 knockout mice (Sirt5-/-) [90] were obtained from the Jackson Laboratory (B6;129-Sirt5tm1Fwa/J, Stock #: 012757). B6/129S F2 strain wild-type (WT) mice were used as controls. Age-matched 10-14 weeks old male mice were used throughout the study. Ischemic-AKI was induced by a renal ischemia-reperfusion injury (IRI) model as previously descried [91] with modifications. Briefly, mice were anesthetized with inhalant 2% isoflurane. Core body temperature of the mice was monitored with a rectal thermometer probe and was maintained at 36.8-37.2°C throughout the procedures with a water heating circulation pump system (BrainTree Scientific, EZ-7150) and an infrared heat lamp (Shat-R-Shield). Buprenorphine (Par Pharmaceutical) was administered for pain control (0.1 mg/kg bw, s.c.). With aseptic techniques, a dorsal incision was made to expose the kidney. 22 minutes renal ischemia was induced by unilateral clamping of the left kidney pedicle with a non- traumatic microvascular clamp (Fine Science Tools, 18055-04). Renal reperfusion was visually verified. Delayed contralateral nephrectomy of the right kidney was performed at day 6 [92]. Mice were sacrificed at day 7 to harvest blood and the injured kidney. Serum was separated from the blood and analyzed by the Kansas State Veterinary Diagnostic Laboratory for levels of creatinine and blood urea nitrogen (BUN).

To induce cisplatin-AKI, mice were given a single dose of 20mg/kg i.p cisplatin (Fresenius Kabi) or vehicle control of normal saline as described previously [93]. Mice were sacrificed on day 3 to harvest blood and the kidneys.

2.2 Cultured proximal tubular epithelial cells

Primary mouse proximal tubular epithelial cells (PTEC) were isolated from single-cell kidney suspension of 10-14 weeks old, male, Sirt5-/-, B6/129 strain WT, or 129 strain WT mice by Dynabeads™ FlowComp™ Flexi Kit (Thermo Fisher Scientific) conjugated to Lotus Tetragonolobus Lectin (LTL) (Vector Laboratories, L-1320). Passage 3-6 PTEC were used for experiments. Human kidney proximal tubular epithelial cells (hPTEC) were obtained from ATCC (Manassass, VA). Both types of cells were cultured as previously described with modifications.

Mouse primary PTEC were plated at 10^6 cells per well and, under serum-restricted conditions, (1) exposed to 24 hours of normoxia or hypoxia (FiO2 1%) in the hypoxia chamber (Coy Laboratory) or (2) treated with 24 hours 20μ M cisplatin or vehicle control of normal saline.

Human proximal tubular cells (hPTEC) (ATCC PCS-400-010), were maintained in a 1:1 mix of Dulbecco's modified Eagle's medium (Thermo Fisher Scientific) and Ham's F12 (Thermo Scientific) containing 5mM glucose and supplemented with 2mM GlutaMAX (Thermo Scientific), 1X Insulin-Transferrin-Selenium (Gibco), 36 ng/mL hydrocortisone, and 0.1% (v/v) penicillin/streptomycin at 37°C in 5% CO2.

To knockdown Sirt5, the plasmid containing Sirt5 siRNA (Ambion, 16708) and the control plasmid (scrambled, Thermo Fisher Scientific, AM4611) were reverse-transfected into hPTEC by using INTERFERin transfection reagent (Polyplus, 409-10) according to the manufacturer's protocol. hPTEC were used for experiments 48 hours after siRNA treatment. To inhibit ACOX1 activity, 500nM 10,12-tricosadiynoic acid (TDYA, Sigma) is used [94].

2.3 Combined Glucose/Oxygen Deprivation (CGOD)-mediated *in vitro* ischemic AKI

hPTEC were subject to an in vitro ischemic-AKI model by combined glucose/oxygen deprivation (CGOD) insult. For the CGOD procedure, hPTEC in a 24-well or 96-well plates were exposed to 4 to 24 hours of normoxia or hypoxia (FiO2 1%) with defined glucose-free buffer (115 mM NaCl, 1.0 mM NaH2PO4, 26.2 mM NaHCO3, 5.4 mM KCl, 1.8 mM CaCl2,

and 0.8 mM MgSO4). After CGOD procedure, culture medium was replaced with glucose-containing buffer (+30mM glucose) and hPTEC moved to a regular incubator for normoxic treatment in the next 24 hours to simulate "reperfusion". The defined glucose-free buffer was used instead of glucose-free cell culture media because other components in medium, such as amino acids, can alter the response of cells to hypoxia [95, 96].

2.4 Lactate Dehydrogenase release assay

Cell death was assessed by lactate dehydrogenase (LDH) efflux using the LDH release assay kit (Promega) in accordance with the manufacturer's protocol. Cytotoxicity was expressed as the ratio of the LDH release in the treated cell medium to that of the maximal LDH release.

2.5 Western Blots

Kidney lysates were lysed in radioimmunoprecipitation assay buffer (RIPA buffer; Thermo Fisher Scientific) and the homogenates plated in triplicate to measure protein concentration using

a Bradford assay kit (Bio-Rad). The following antibodies were used in this study; Anti-Acox1 antibodies (1:1000, Rabbit, Abcam, ab59964), Pan-succinyllysine antibodies (1:1000, Rabbit, PTM Biolabs, PTM-401), Anti-Sirt5 antibodies (Rabbit, 1:50, Cell Signaling, 8782). Anti-GAPDH or Anti-α/β-tubulin were used as loading controls at 1:100.

2.6 Real-time PCR

Real-time PCR analysis was performed as previously described [97], to determine mRNA level in whole kidneys. cDNA was reverse-transcribed from 500 ng of total RNA with SuperScript First-Strand Synthesis System (Thermo Fisher Scientific). Real-time PCR analysis was performed with gene specific primer oligos, SsoAdvanced™ SYBR® Green Supermix (Bio- Rad), and CFX96 Touch™ Real-Time PCR Detection System with C1000 Thermal Cycler (Bio- Rad). Cycling conditions were 95 °C for 10 minutes, then 40 cycles of 95 °C for 15 seconds and 60 °C for 1 minute. Rn18S was used for endogenous control. Expression was compared to Rn18S endogenous control and analyzed using the 2 -ΔΔCt method.

2.7 Tissue section analysis

Kidneys were fixed in 4% paraformaldehyde and embed in paraffin. These tissues were sectioned at 4 microns. The kidney sections were stained with hematoxylin and eosin (H&E) or Masson's Trichrome. H&E stained slides were subject to histological evaluation. 40x images of renal cortex and medulla were obtained with a Leica DM2500 optical microscope. Semi-

quantitative scoring (0-4) for tubular injury was performed in terms of tubular dilation, proteinaceous cast formation, and loss of brush border. To quantify Masson's trichrome stained, collagen-rich area, tiling imaging system via TissueFAXS PLUS (TissueGnostics) were used to capture the entire kidney section with 20x objectives. Masson's Trichrome positive area was quantified with NIS Elements software (Nikon). All tissue evaluation was performed in a blinded fashion.

Immunostaining was performed as previously described with paraffin-embedded tissues and with following primary antibodies or lectins: β-galactosidase (chicken, 1:200, Abcam, ab9361), Endomucin (rat, 1:200, Santa Cruz, sc-65495), Kim1 (rat, 1:100, R&D Systems, MAB1817), NCC (Rabbit, 1:250, Millipore, AB3553), NGAL (rat, 1:50, abcam, ab70287), PMP70 (Rabbit, 1:200, Abcam, ab85550), Dolichos Biflorus Agglutinin (DBA) (1:200, Vector Laboratories, FL-1031), or LTL (1:200, Vector Laboratories, FL-1321).

2.8 Palmitate oxidation assay

^{14}C-palmitate (PerkinElmer) was conjugated to BSA and used at 125 μM in 200μl reactions as described [65]. ^{14}CO2 was captured on filter papers soaked in 1M KOH and water- soluble 14C-labeled FAO products were separated by the method of Bligh and Dyer [98] and subjected to scintillation counting. Peroxisomal FAO was defined as the rate of palmitate oxidation in the presence of the irreversible mitochondrial FAO inhibitor etomoxir (100 μM). Data were normalized to protein concentration.

2.9 Oroboros high-resolution respirometry

Freshly-prepared kidney homogenates were analyzed with an Oroboros Oxygraph-2K using our previously published method [99]. Complex I respiration was defined as malate/pyruvate/glutamate-driven oxygen consumption in the presence of ADP, while Complex II respiration was defined as succinate-driven oxygen consumption.

2.10 Quantitative mass spectrometry

Both 7-day renal IRI- and contralateral control- kidney tissues (n=3) were homogenized, trypsinized, and then succinylated peptides were enriched using the PTMScan Succinyl-Lysine Motif Kit (Cell Signaling Technologies). Following reverse-phase HPLC-ESI-MS/MS, succinyl enriched samples were analyzed by dataindependent acquisition (DIA, e.g. SWATH) on a TripleTOF 6600, and site-specific changes in succinylation were quantified using Skyline as described [100-102].

3.0 Results and Discussion

3.1 Sirt5-/- kidneys exhibit protein lysine hyper-succinylation

Sirt5 is highly expressed in kidney [73] but its role in kidney function has not been examined. I confirmed that global Sirt5 knockout mice (Sirt5-/-) have no detectable Sirt5 mRNA in the kidney (Fig 2A). Similar to liver and heart, Sirt5 deletion in the kidney leads to accumulation of post translation modification (PTM) known as lysine succinylation shown by a pan anti-succinyllysine western-blotting for whole kidney lysates (Fig 2B). The mutant allele in Sirt5-/- mice bears a lacZ reporter cassette [90] . β-galactosidase (β-gal) immunostaining revealed that Sirt5-β-gal is co-localized in Lotus tetragonobolus lectin (LTL)-positive PTEC (Fig 2C), in Thiazide-Sensitive NaCl Cotransporter (NCC)-positive distal tubular epithelial cells and Dolichos Biflorus aggluttanin (DBA)- positive collecting ducts but was absent from Endomucin- positive microvasculature (Not shown). In wild-type (WT) mice, Sirt5 mRNA was expressed in the whole kidneys and was enriched in isolated primary PTEC (Fig 2D). At baseline, Sirt5-/- kidneys appear histologically (Fig 2E, F) and functionally normal with unaltered serum creatinine and blood urea nitrogen (BUN) compared to WT (Fig. 2F).

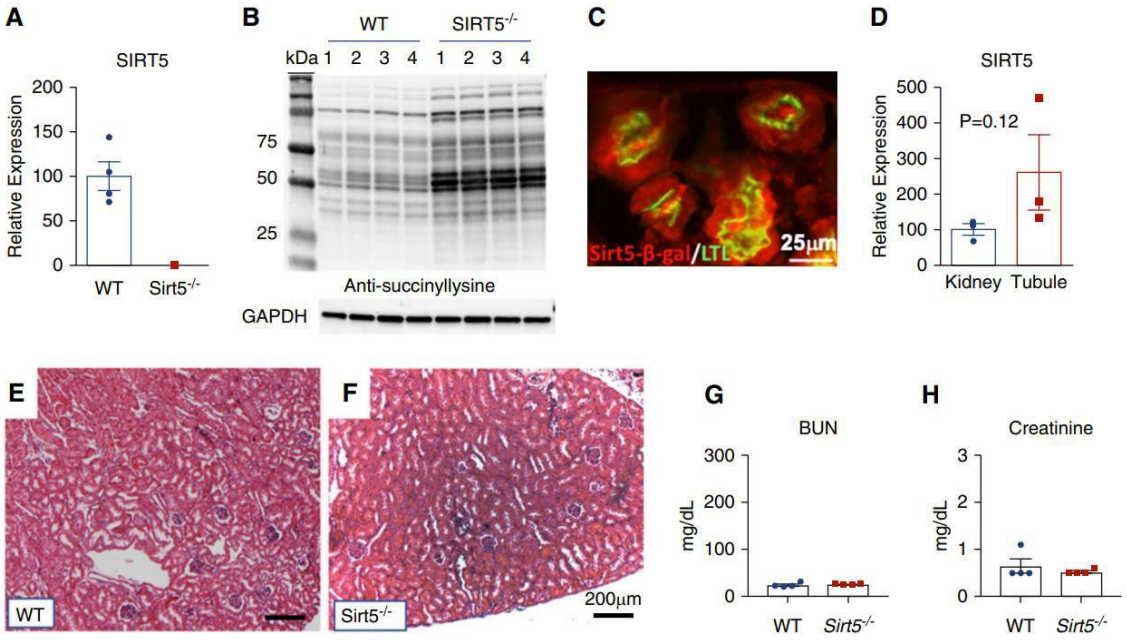


Figure 2: Sirt5-/- kidneys exhibit protein lysine hyper-succinylation but normal morphology and function. Real-time PCR of Sirt5-/- kidney confirms no detectable Sirt5 mRNA in mouse kidney (n=4/group). B. Western-blotting of kidney lysates (50 µg) with anti-succinyllysine antibody was used to assess succinylation levels in mouse kidneys derived from wild-type (WT) or Sirt5-/- mice (n=4/group). C. The Sirt5-/- mutant allele contains a lacZ cassette that allows for the interrogation of expression using an anti-β-galactosidase (β- gal) antibody (Sirt5-β-gal, red). Abundant expression of Sirt5 is co-localized in the proximal tubular epithelial cells (LTL, green). Scale bar = 25µm. D. Real-time PCR of whole kidney compared with isolated LTL+ proximal tubular epithelial cells (PTEC) suggest that Sirt5 expression is enriched in PTEC (n=3/group). E-F. H&Estained kidneys of wild-type (WT) and Sirt5-/- kidneys at baseline. No discernable differences were noted between WT and Sirt5-/- kidneys. Scale bar = 200µm. G, H. Serum levels of (G) BUN and (H) creatinine were not significantly different at baseline between WT and Sirt5-/- mice (n=4/group). All data are presented as dot plots plus mean ± SEM. t-test

3.2 Sirt5-/- kidneys are protected against ischemic-AKI in mice

To test the role of Sirt5 in ischemic-AKI, Sirt5-/- mice were subjected to a renal ischemia-reperfusion (IR) mediated ischemic-AKI model and were sacrificed 7 days later. H&E- stained histopathology revealed decreased proteinaceous casts, less severe tubular dilation, and reduced loss of brush border in Sirt5-/- kidneys (Fig 3A, D, G). Kidney injury molecule-1 (Kim- 1), which localizes to injured PTEC [103], was much more prominent in WT kidney (Fig 3B versus 3E). Further, neutrophil gelatinase-associated lipocalin (NGAL), another marker for kidney tubular injury[104], was observed in many WT but was virtually absent in Sirt5-/- PTEC (Fig 3C, C' versus F, F'). Real-time PCR for Lcn2 (NGAL mRNA) confirmed its decreased mRNA level in Sirt5-/- kidney (Fig 3J). Finally, kidney function was protected in Sirt5-/- mice as evidenced by reduced serum creatinine and BUN (Fig. 3H, I). Interestingly, similar results were observed by using the heterozygous mutant (Sirt5+/-) and their littermate WT controls, suggesting that half of Sirt5 gene dosage is sufficient to protect against ischemic-AKI.

PTEC are sensitive to ischemic- and cisplatin-AKI, and are reliant upon mitochondria for energy metabolism; Sirt5 is known to regulate mitochondrial function and is enriched in PTEC (Fig 2). Therefore, I hypothesized that the protection against ischemic-AKI in Sirt5-/- was occurring within PTEC. To test this, primary PTEC were isolated from Sirt5-/- and B6/129-strain WT mice and exposed to 24-h, 1% hypoxic insult in vitro. Following the hypoxia exposure, expression of the tubular injury markers, Havcr1 (Kim-1 mRNA), Lcn2, and Interleukin-18 (IL-18) was significantly reduced in Sirt5-/- PTEC (Fig 4A). Sirt5-/- mice are a mixed background of the C57Bl/6 and 129Sv strains, and 129Sv-strain mice have been shown to exhibit resistance to ischemic-AKI [105]. To determine if this is the case in cultured mouse PTEC, I

subjected PTEC from WT B6/129, WT 129, and Sirt5-/- mice to 24 h of 1% hypoxia. The results confirm that (1)

WT 129 is more protective than WT B6/129, evidenced by reduction of Havcr1 and Lcn2 mRNA and that (2) Sirt5-/- is more protective than WT 129, shown by reduction of Havcr-1, IL-18, and Lcn2 mRNA (Not shown). I further modeled ischemic-AKI in vitro with primary human PTEC (hPTEC) using a combined glucose/oxygen deprivation (CGOD) protocol [106]. In this protocol, glucose and oxygen are deprived for the first 24-h and then added back to "reperfuse" for an additional 24-h. A siRNA knockdowns Sirt5 levels, with an efficiency of ~80%, when compared to a scrambled siRNA control (Fig 4C). At the end of the 24-h CGOD and the subsequent 24-h "reperfusion" period, hPTEC with Sirt5 knockdown demonstrated protection as evidenced by reduced lactate dehydrogenase (LDH) efflux (Fig 4D).

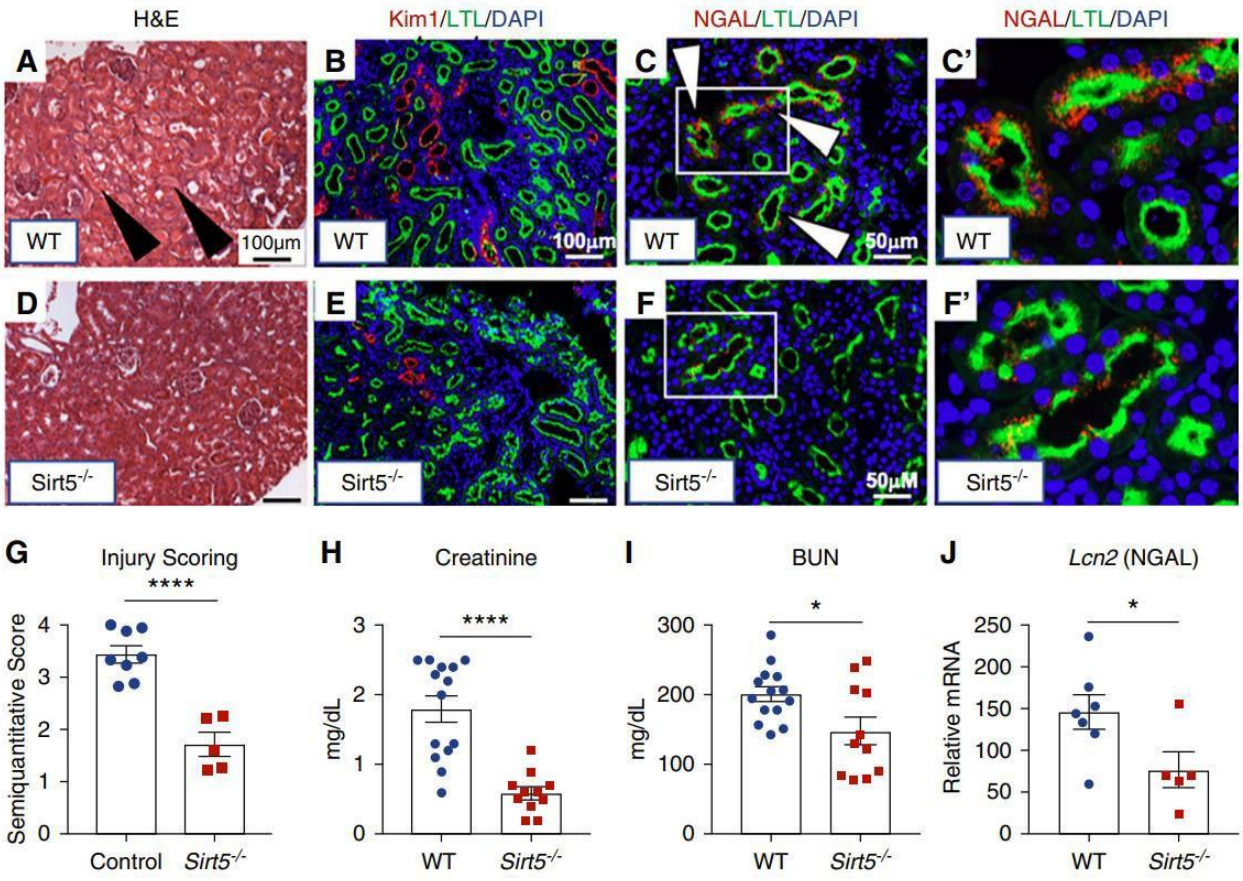


Figure 3: Sirt5-/- kidneys are protected against ischemic-AKI at 7 days following injury. A, D, G. H&Estained kidneys of (A) wild-type (WT) and (D) Sirt5-/- kidneys that were subjected to the ischemic-AKI model. The Sirt5-/- kidneys had decreased injury score as compared to WT kidneys, evidenced by proteinaceous casts in WT (arrow heads), dilated tubular structures, and vacuolization (arrows) that were decreased in Sirt5-/- kidneys (n=5-8/group) Scale bar = 100µm. B and E. Immunostaining for kidney injury molecule 1 (Kim1, red) in the proximal tubular epithelial cells (PTEC) (LTL, green) showed a large increase in the amount of Kim1 expression in the (B) WT compared to very low levels in the (E) Sirt5-/- PTEC. Scale bar = 100µm. C,C'(inset) and F,F'(inset). Immunostaining for neutrophil gelatinase-associated lipocalin (NGAL, red) in PTEC (LTL) was observed in many WT while virtually absent from Sirt5-/- PTEC. Scale bar = 50µm. J. Real-time PCR for Lcn2 (NGAL mRNA) confirms the decrease of Lcn2 in Sirt5-/- kidneys compared to WT (n=5-7). H and I. Serum levels of (I) Creatinine and (J) BUN are decreased in the Sirt5-/- mice compared to WT, suggesting protected kidney function in the Sirt5-/- mice (n=11-14/group). All data are presented as dot plots plus mean ± SEM. *p<0.05, ****p<0.0001, t-test

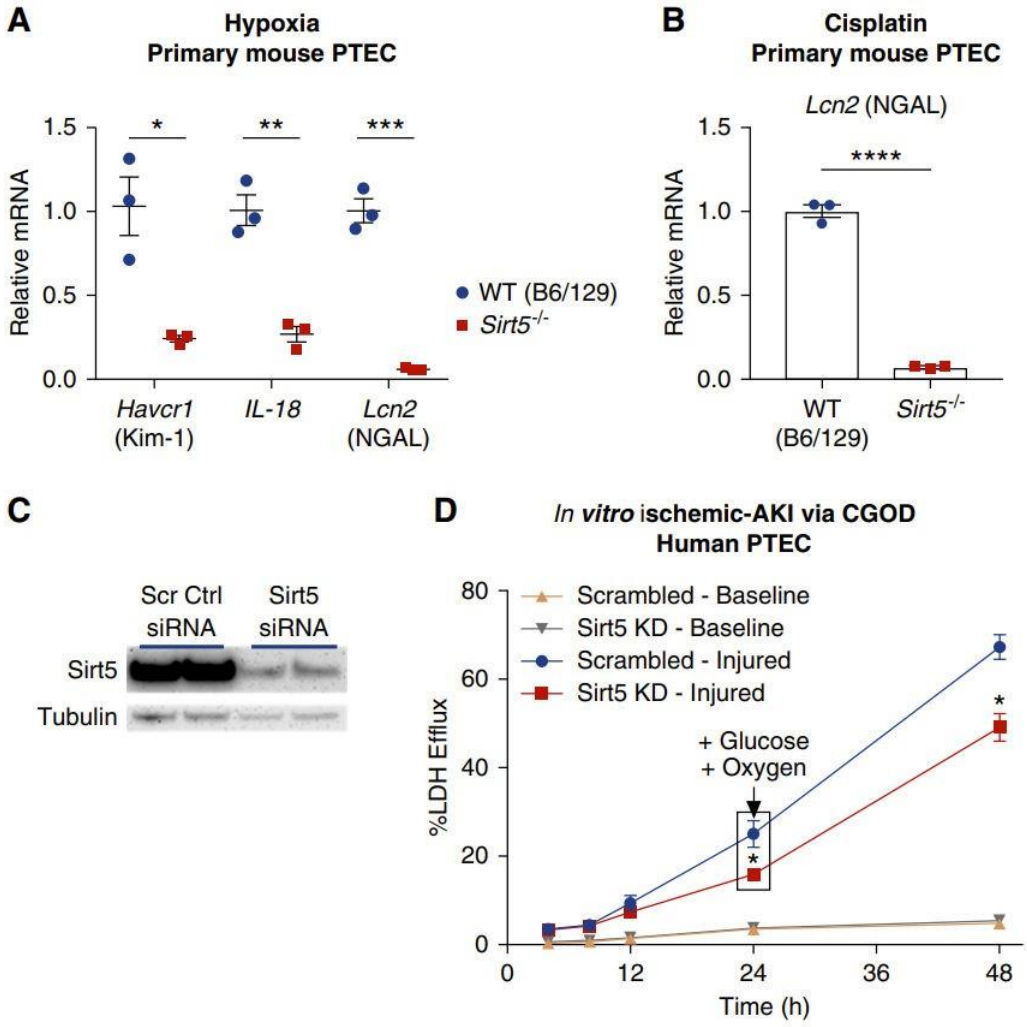


Figure 4: Loss-of-function of Sirt5 in proximal tubular epithelial cells is protective against injury in vitro. A. 24-h 1% hypoxia-exposed primary proximal tubular epithelial cells (PTEC) derived from Sirt5-/- kidneys exhibited reduced mRNA levels of injury markers Havcr1 (Kim1 mRNA), Lcn2 (NGAL mRNA), and IL18, compared to B6/129-strain wild-type (WT) PTEC (n=3/group). B. 24-h Cisplatin-treated primary mouse PTEC from Sirt5-/- kidneys exhibited reduced mRNA levels of Lcn2, compared to WT PTEC (n=3/group). C. Western blotting confirms siRNA knockdown (KD) of Sirt5 in primary human PTEC (hPTEC). D. hPTEC is subjected to combined glucose/oxygen deprivation (CGOD)-mediated in vitro ischemic-AKI model; E. Sirt5 KD hPTEC showed a marked decrease in lactate dehydrogenase (LDH) release (n=3/group). All data are presented as dot plots plus mean ± SEM. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, t-test

3.3 Sirt5-/- mice are protected against cisplatin-induced AKI

To determine if the protection afforded by Sirt5 loss-of-function extends to other forms of AKI, Sirt5-/- mice were challenged with a high dose (20 mg/kg bw i.p) of the nephrotoxin cisplatin and were sacrificed 3 days later. Cisplatin specifically targets PTEC. H&E-stained kidney tissues demonstrated less severe tubular injury in cisplatin-treated Sirt5-/- mice compared to WT mice (Fig 5A-C). Kidney function was also protected in Sirt5-/- mice as evidenced by reduced serum creatinine and BUN (Fig 5D, E). During the 72-h post cisplatin treatment, WT mice lost 14.5% of their body weight compared to just 8.4% among Sirt5-/- mice (Fig 5F), suggestive of a blunted response to cisplatin in the Sirt5-/- mice. Real-time PCR analysis for Havcr1 and Lcn2 confirmed decreased mRNA level of the tubular injury markers in Sirt5-/- kidney (Fig 5G). Consistent with this, many WT PTEC were co-localized with NGAL while this was not observed in Sirt5-/- PTEC (Fig 5H-I). Renoprotection was also confirmed in the PTEC as evidenced by the strong expression of Lcn2 in WT primary PTEC treated with 20 μM cisplatin in vitro as compared to Sirt5-/- PTEC (Fig 5B). Finally, cultured primary PTEC derived from heterozygous Sirt5+/- mice reduced mRNA level of Lcn2 after 24-h cisplatin treatment when compared to the PTEC derived from their littermate WT controls (Not shown).

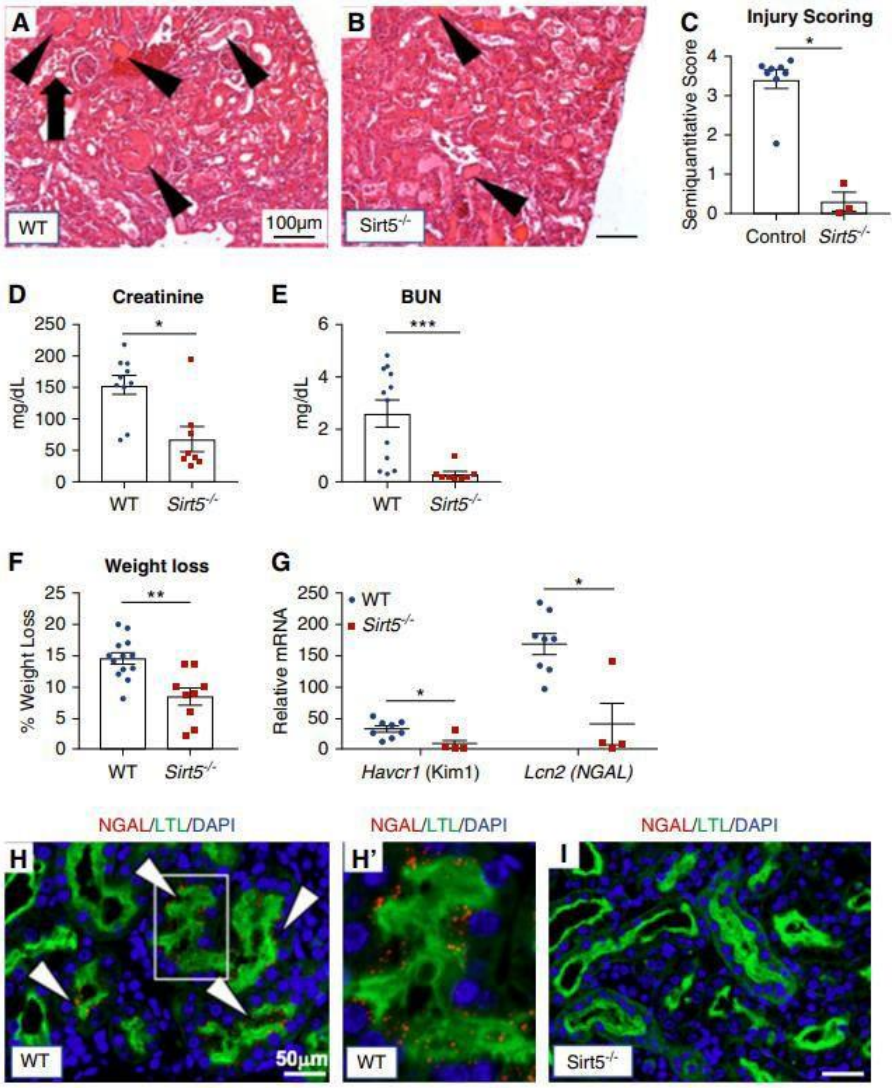


Figure 5: Sirt5-/- kidneys are protected against cisplatin-induced AKI at 3 days following injury. A-C. H&E-stained kidneys of (A) wild-type (WT) and (B) Sirt5-/- kidneys that were subjected to the cisplatin-AKI model. The Sirt5-/- kidneys had decreased injury kidneys compared to WT (n=4-8). Mann–Whitney U test. H-J. WT and Sirt5-/- kidneys were immunostained for NGAL (red) and proximal tubular epithelial cells (PTEC) (LTL, green). WT kidneys (H-H') had increased NGAL positive (arrows, red) in LTL-positive PTEC than Sirt5-/- kidneys (I). Scale bar = 50μm. All data are presented as dot plots plus mean ± SEM. *p<0.05, **p<0.01, ****p<0.0001

3.4 Total FAO is increased in Sirt5-/- kidneys both at baseline and after ischemic-AKI

A lysine succinylome analysis implicated FAO as a key pathway to confer renoprotection in Sirt5-/- kidneys. This pathway is known to be suppressed in Sirt5-/- liver and heart [87, 107]. To explore the effects of Sirt5 loss-of-function on kidney FAO, I followed the catabolism of 14C-labeled palmitate to $^{14}CO2$ in fresh kidney lysates prepared from uninjured kidneys. Contrary to expectation, baseline FAO rates were significantly elevated in Sirt5-/- kidneys (Fig 6A). This was not due to a greater number of mitochondria, as quantification of the Tomm20- positive mitochondria (Fig 6B) and the mitochondrial: nuclear DNA ratio (Fig 6C) indicated similar mitochondrial abundance across genotypes. Consistently, baseline FAO rates were trending to be increased ($p=0.10$) in cultured Sirt5-/- PTEC (Not shown). Our group also analyzed respiratory chain function, previously shown to be impaired in Sirt5-/- liver mitochondria [99], in kidney lysates using an Oroboros high-resolution respirometer. As in liver, uninjured Sirt5-/- kidney mitochondria demonstrated a significant decrease in respiration on the Complex II substrate succinate (Fig 6D, E).

I next repeated the same set of experiments on Sirt5-/- and WT kidney tissue isolated Day 7 postrenal IRI. FAO rates, overall, were an order of magnitude lower in kidneys subjected to 22-min renal IRI compared to baseline (compare Fig 6F to 6A). A more severe 30-min renal IRI reduced FAO rates even further.

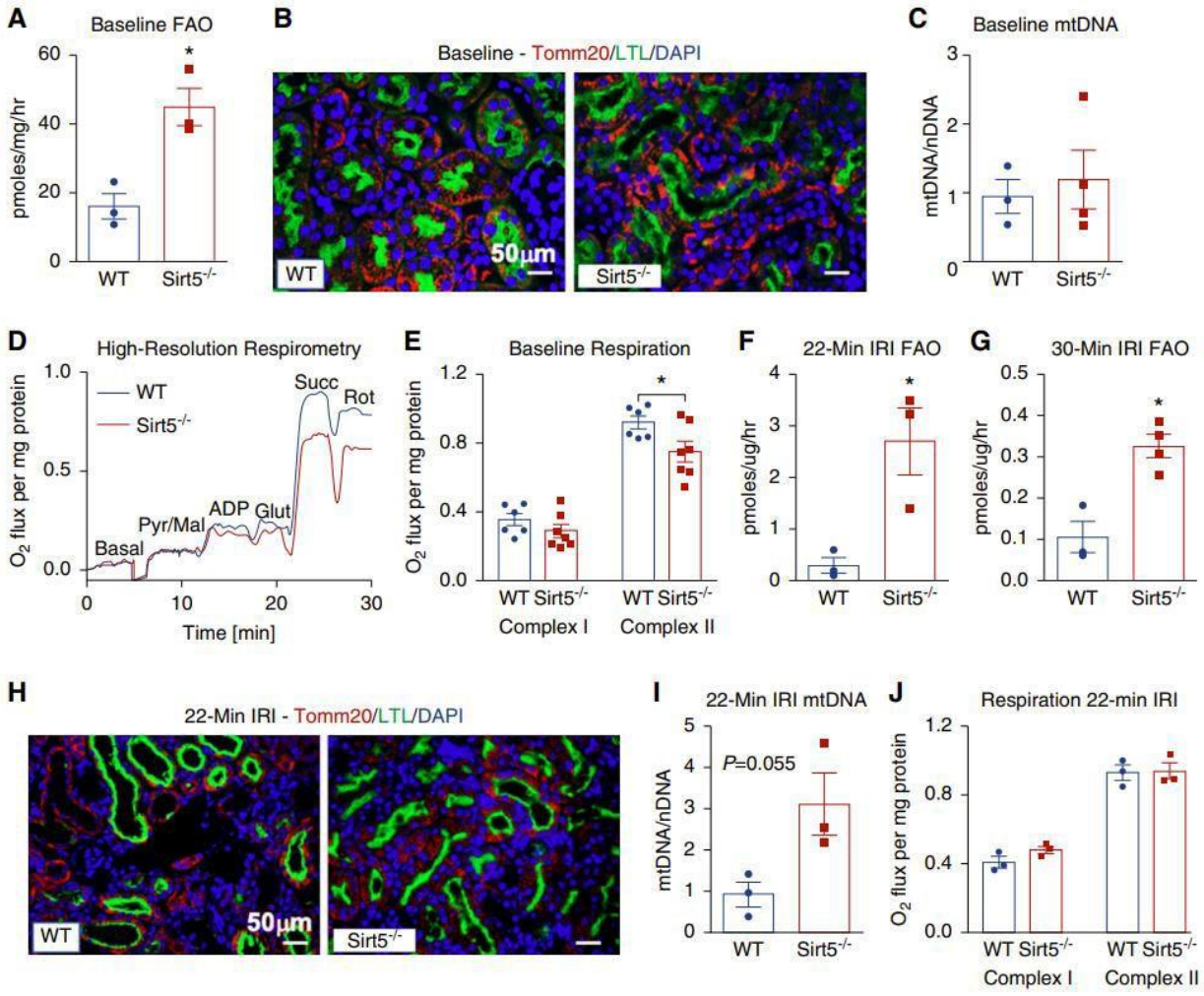


Figure 6: Fatty acid oxidation (FAO) is enhanced in Sirt5-/- kidneys. A. Total FAO is increased at baseline in whole kidney homogenates of Sirt5-/- mice compared with wild-type (WT), as measured by 14C-palmitate oxidation. (n=3/group) B. Immunostaining reveals equivalent numbers of Tomm 20 positive mitochondria (red) in WT and Sirt5-/- proximal tubular epithelial cells (PTEC) (LTL, green) at baseline. Scale bar = 50µm. C. Mitochondrial DNA (mtDNA) measured by real-time PCR was found to be equivalent between control and Sirt5-/- kidneys (n=3-4/group). D-E. Baseline respirometry revealed that complex II specifically and not complex I of the electron transport chain was reduced in Sirt5-/- kidneys compared with WT (n=6-7/group). F-G. After a moderate (22-min) (F) or severe (30-min) (G) renal ischemia reperfusion injury (IRI), Sirt5-/- kidneys displayed an increased rate of FAO (n=3-4/group) H. After renal-IRI mitochondria (Tomm 20, red) appear to be present in both WT and Sirt5-/- proximal tubules (LTL, green). Scale bar = 50µm. I. Mitochondrial DNA trended to be increased in Sirt5-/- kidneys compared with WT. (n=3/group) J. No

difference was observed in complex I or Complex II utilization of the electron transport chain after renal-IRI. (n=3/group). All data are presented as dot plots plus mean ± SEM. *p<0.05, t-test

3.5 Increased FAO in Sirt5-/- kidneys is localized to peroxisomes

There are two parallel pathways of FAO, one in mitochondria that directly transmits reducing equivalents into the electron transport chain and acetyl-CoA into the TCA cycle, and one in peroxisomes that partially chain-shortens long-chain fatty acids and releases short-chain products into the cytosol. These short-chain products can either be taken up by mitochondria and metabolized to completion or released from the cell. PTEC contain an abundance of peroxisomes and increased peroxisomal function has been previously linked to renoprotection [108, 109]. Recently, peroxisomal gain-of-function was reported in Sirt5-/- cell lines and mouse liver [87, 99, 110]. I therefore interrogated Sirt5-/- kidneys for peroxisomal FAO at baseline and following renal IRI. The catabolism of 14C-palmitate was followed to $^{14}CO2$ and water-soluble (short- chain) products in kidney homogenates in the presence of etomoxir, an irreversible inhibitor of mitochondrial FAO. Etomoxir-resistant FAO is ascribed to peroxisomes [25] . Sirt5-/- kidney homogenates at baseline displayed a significantly higher rate of peroxisomal FAO than WT kidney homogenates (Fig 7A). A similar result was obtained using freshly isolated primary mouse PTEC (Fig 7B) and a similar trend was observed using cultured hPTEC with Sirt5 siRNA knockdown (Not shown). After a severe 30-min renal IRI, peroxisomal FAO was 7-fold higher in Sirt5-/- kidney homogenates (Fig 7C). The 30-min renal IRI homogenates were western- blotted for acyl-CoA oxidase-1 (ACOX1), the rate-limiting enzyme of peroxisomal FAO. The abundance of ACOX1 was significantly higher in Sirt5-/- kidney (Fig 7D). Sirt5-/- kidneys were confirmed to

have greater abundance of Peroxisomal Membrane Protein 70 (Pmp70)-positive peroxisomes in baseline and post-renal IRI tissues (Fig 7E, F). To test whether inhibition of peroxisomal FAO abrogated the beneficial effect of Sirt5 inhibition, we used Sirt5 siRNA in cultured human PTEC ± 10,12-tricosadiynoic acid (TDYA), an irreversible peroxisomal FAO inhibitor, during in vitro ischemic-AKI (Fig 7G). The combined Sirt5/peroxisomal FAO inhibition removed the protective role of Sirt5 knockdown confirming the role of peroxisomal FAO switching in driving the PTEC protection. These data confirm that Sirt5-/- kidneys undergo an FAO switch from mitochondria to peroxisomes.

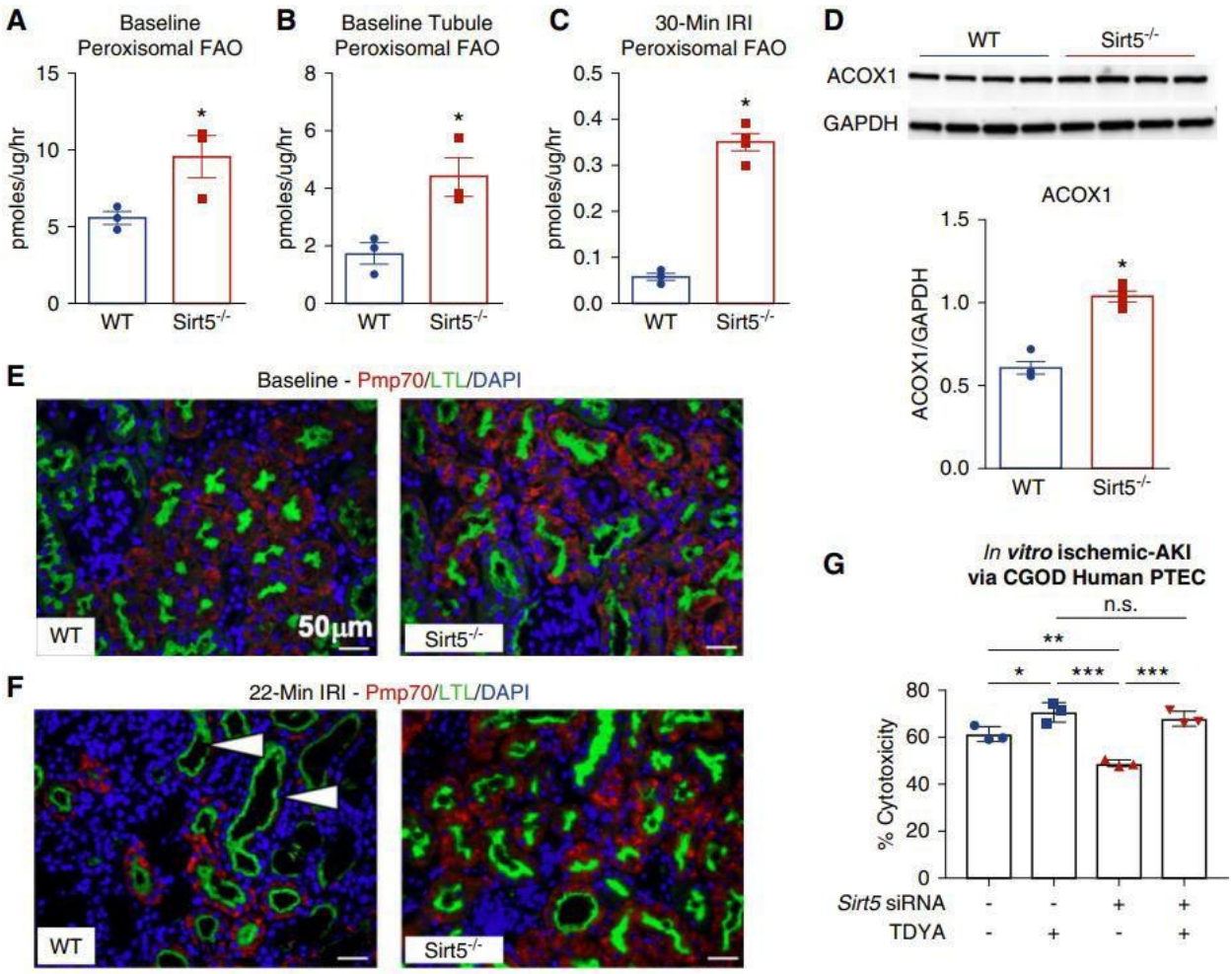
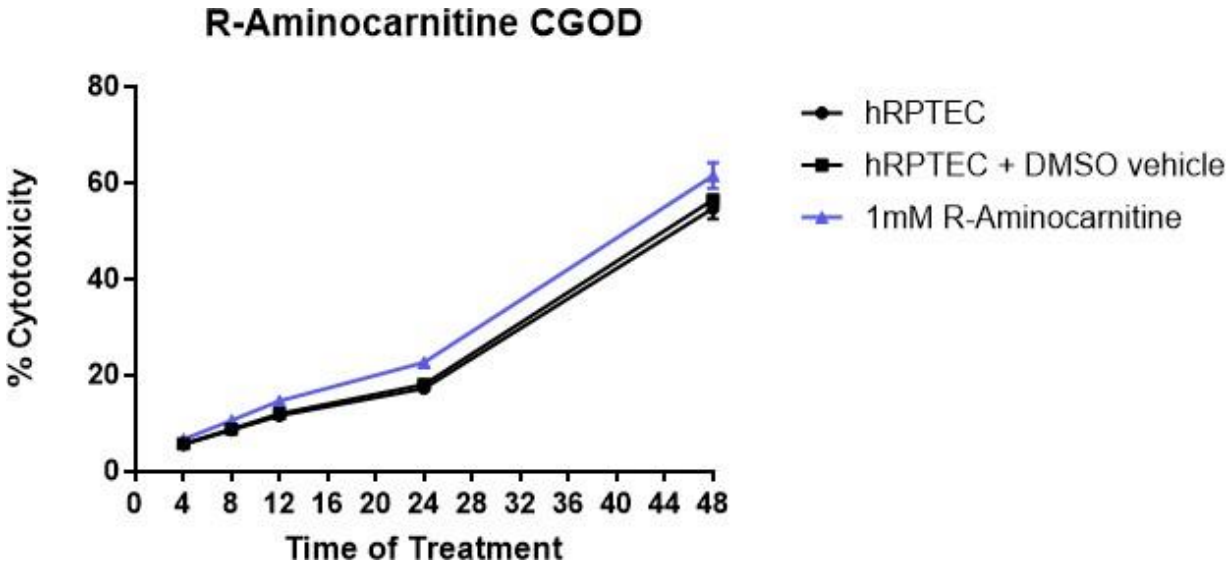


Figure 7: Increased FAO by Sirt5 loss-of-function is localized to peroxisomes. A-C. Etomoxir-insensitive 14C-palmitate oxidation, which represents peroxisomal FAO, was measured in kidney homogenates at baseline (n=3/group) (A), in freshly isolated proximal tubular epithelial cells (PTEC) (n=3/group) (B), or after 30-minute renal-IRI (n=3/group) (C). t-test D. Western blot analysis showed an upregulation of the rate-limiting peroxisomal FAO enzyme ACOX1 in Sirt5-/- kidney homogenates. Mann–Whitney U test. E-F. PMP70 (red) is a peroxisome marker, LTL (green) is a marker for PTEC and DAPI (blue). E=baseline, F=post renal IRI. (n=4/group) E and F. At baseline (E) there appear to be more peroxisomes present in the proximal tubules (arrows) and this persists after injury. Scale bar = 50µm. (F). G. Human PTEC (hPTEC) was treated ± Sirt5 siRNA and ± TDYA (ACOX1 inhibitor) during combined glucose/oxygen deprivation (CGOD)-mediated in vitro ischemic-AKI and was evaluated its effect by LDH release (cell death marker). TDYA treatment

abrogates the protective effect by Sirt5 siRNA. (n=3/group). Tukey comparison. All data are presented as dot plots plus mean ± SEM. *p<0.05, **p<0.01, ***p<0.001

3.6 Pharmacological agents to modulate metabolism and AKI *in vitro*

Given the finding that deletion of SIRT5 is renoprotective, another therapeutic avenue exists for selective sirtuin inhibitors. In the case of SIRT5, which uniquely targets succinyl modifications, inhibitors targeting the succinyl substrate are current under development [111, 112]. However, the development of sirtuin inhibitors holds some risk as sirtuins are involved in myriad pathways, and whether modulating sirtuins will have a beneficial or deleterious effect in humans is unclear. The compromise to this double-edged sword might be the development of therapeutics targeting the pathways shown to be regulated by sirtuins rather than the sirtuins themselves. To that end, I tested the therapeutic efficacy of various metabolic modulators *in vitro* for their effect on cell injury using a lactate dehydrogenase (LDH) release assay (Figures 8-11). Although Etomoxir, an irreversible inhibitor of Cpt1, has been shown to protect against kidney injury. Teglicar, a reversible inhibitor of Cpt1, accelerated release of LDH and exacerbated injury (Figure 8). Addition of pharmacological agent C75, a fatty acid synthase inhibitor, also exacerbated injury especially 12 hours after treatment (Figure 9). R-aminocarnitine, an inhibitor of Cpt2 (while not acting as a PPAR-activator), had only slightly elevated release of LDH compared to DMSO control (Figure 10). Finally, bezafibrate, a PPARα activator, showed a modest reduction in LDH release over 48 hours compared to DMSO control (Figure 11).


R-Aminocarnitine CGOD
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Time of Treatment
hRPTEC
hRPTEC + DMSO vehicle
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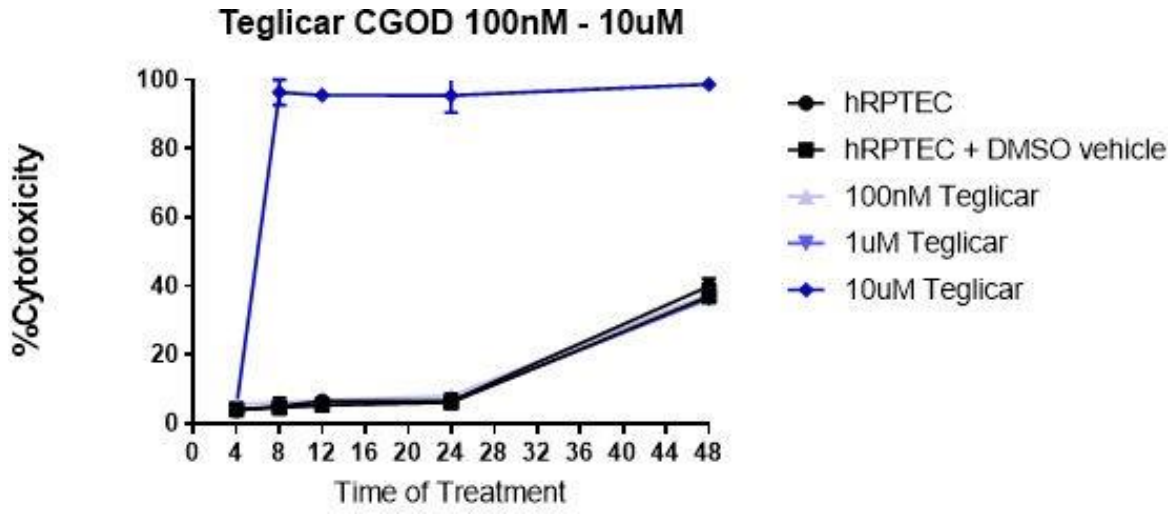
Teglicar CGOD 100nM - 10uM
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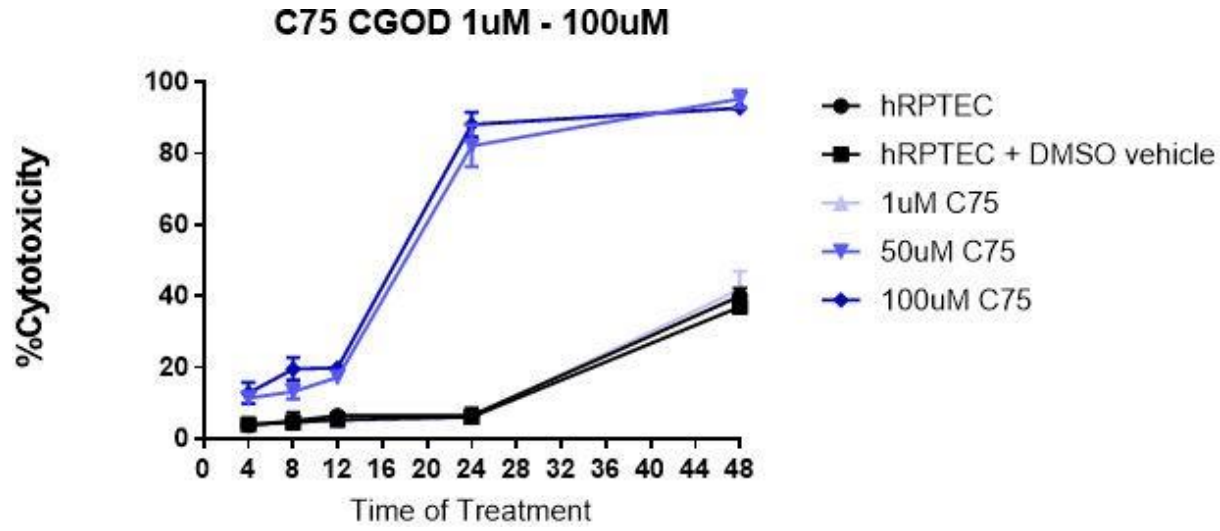
C75 CGOD 1uM - 100uM
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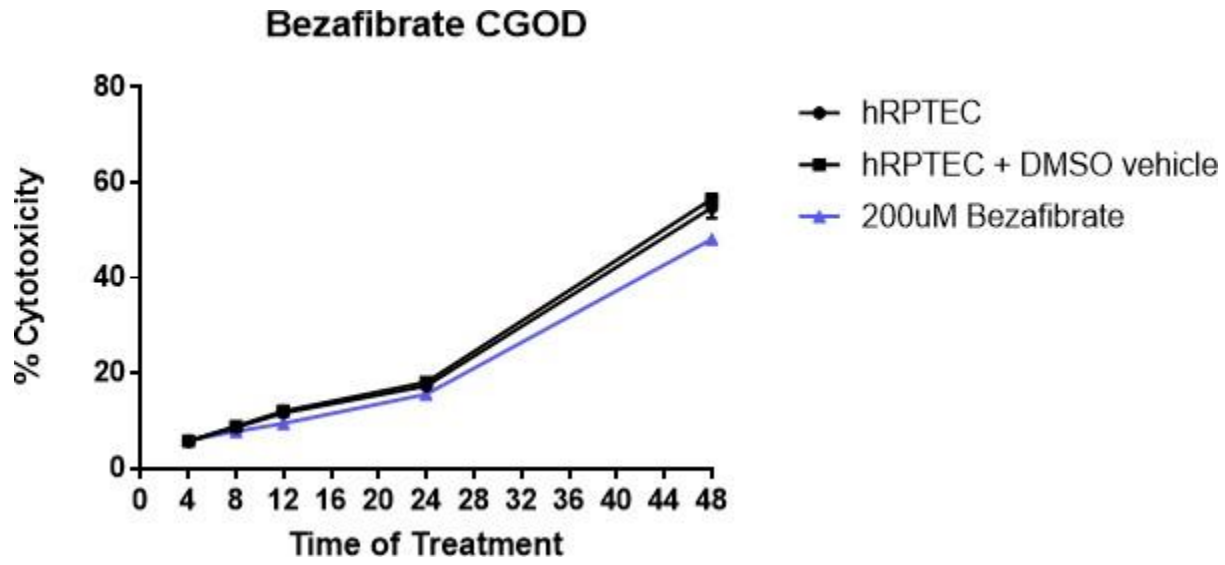

Figure 8: Effect of various metabolic modulators on the release of lactate dehydrogenease in hRPREC cells. A. hPTEC showed a marked increase in lactate dehydrogenase (LDH) release compared to DMSO control when given Teglicar, an irreversivle inhibitor of Cpt1 (n=3/group). B. hPTEC showed a sharp increase in lactate dehydrogenase (LDH) release compared to DMSO control when given C75, an inhibitor of fatty acid synthase. C. hPTEC showed a moedest increase in lactate dehydrogenase (LDH) release compared to DMSO control when given R-aminocarnitine, an inhibitor of Cpt2 (n=3/group). D. hPTEC showed a modest decrease in lactate dehydrogenase (LDH) release compared to DMSO control when given bezafibrate, a PPARα activator (n=3/group).

3.7 Discussion

My studies interrogated the role of Sirt5 during AKI and found that loss-of-function of Sirt5 was renoprotective. This finding is opposite to those observed for Sirt1 and Sirt3, for which deletion exacerbates AKI [69, 113]. We propose that the beneficial nature of Sirt5 loss-of- function stems from the unique combination of only a minor loss of mitochondrial function combined with a significant peroxisomal gain-of-function. Deletion of Sirt5 in the kidney caused only a modest

reduction in mitochondrial function at baseline, i.e. ~10% loss of succinate- induced respiration and a suppression of mitochondrial FAO, and following injury, mitochondrial respiratory chain function was the same across genotypes. This could be due to the clear accumulation of protein succinylation in WT mitochondria following injury (731 succinylated peptides versus 489 at baseline), such that more than 50 peptides exhibited significantly higher succinylation in WT kidney compared to Sirt5-/- kidney after renal IRI (Not shown). Protein succinylation in mitochondria is determined by the concentration of succinyl- CoA, which chemically reacts with lysine residues on the surface of proteins, and the countering activity of Sirt5 [114]. I speculate that WT kidneys rely more upon mitochondria during AKI than Sirt5-/- kidneys, causing accumulation of succinyl-CoA and therefore greater protein succinylation, effectively closing the gap in mitochondrial function between WT and Sirt5-/- kidneys. Peroxisomal function, in contrast, remains enhanced in Sirt5-/- kidney following injury. Increased peroxisomal function has previously been linked to renoprotection in mice treated with etomoxir, in transgenic mice overexpressing Sirt1 in the kidney, and in animals treated with fibrates, a class of drugs that promotes peroxisomal proliferation [115] . PTEC is the major site of injury during several forms of AKI, and is rivaled only by the liver in terms of peroxisome abundance [116]. As previously reported for liver [110] we observed a peroxisomal gain-of function, at least for the FAO pathway, in Sirt5-/- kidneys. Peroxisomes are also known to play an important role in the brain [117]. Interestingly, Sirt5-/- mice are protected from ischemic stroke [118], while in the heart Sirt5 deletion exacerbates ischemic injury [119]. The heart contains only "micro-peroxisomes" which are not thought to contribute significantly to FAO. I speculate that the capacity for peroxisomal FAO might explain why the kidney and brain, but not the heart, are privy to the protective effects of Sirt5 deficiency. Also, defective FAO in PTEC is responsible for interstitial fibrosis. We showed

that although mRNA for two fibrosis markers are decreased in Sirt5-/- kidneys, no discernable difference was observed in tissue across genotypes.

3.7.1 Future Directions

Future studies will evaluate whether Sirt5 loss-of-function leads to fibrosis at later time points (28-42 days post AKI) with more severe ischemic insult, known to drive fibrosis [120]. Therapeutically, due to the deleterious effect on the heart, inhibition of Sirt5 would need to be localized to the kidney. Recent advances have been made in targeting drug delivery to the kidney [121, 122]. Although our data in the Sirt5 heterozygous mice is therapeutically promising as a half gene dosage is sufficient to elicit protection and would likely not lead to off target effects. Further, due to the unique substrate specificity of Sirt5, several Sirt5-specific inhibitors have been developed which demonstrate in vitro efficacy [111, 112]. Future experiments should also endeavor to delete the Sirt5 gene and FAO specifically in the proximal tubular compartment and other nephron segments to determine if the renoprotective phenomena stems from the proximal tubular or other nephron compartments. Additional pharmacological inhibition/stimulation targeting relevant metabolic pathways implicated in these studies should also be performed to determine the relative importance of these various pathways to the renoprotection observed herein.

3.7.2 Conclusion

In conclusion, our results demonstrated that Sirt5 loss-of-function ameliorated ischemic- and cisplatin AKI in mice and human due to a switch away from mitochondrial FAO towards

peroxisomal FAO. Our findings uncover an attractive and novel candidate pathway modulated by Sirt5 for the treatment of AKI.

The study of sirtuins in the kidney has led to impressive advances in our understanding of sirtuin targets involved in renoprotection and in the development of a number of different pharmacological interventions that are effective in ameliorating injury in animal models of AKI. However, the promise of these developments is generally tempered by the results of clinical trials in patients with AKI. Therapies effective in animal models of AKI have translated to little or no effectiveness in humans. However, this failing might stem from deficiencies in preclinical models of AKI and an ability to design the clinical trial itself [123]. Although many auspicious sirtuin targets have been identified, the failure to effectively translate animal data to an effective human intervention highlights the importance of studying AKI in multiple model systems. In addition to the multiple *in vivo* models of AKI available, including the ischemia-reperfusion model, cisplatin-induced AKI, and sepsis-associated AKI, multiple *in vitro* models are used to study AKI [124, 125]. The mechanistic differences between these various models adds to the complexity of AKI pathogenesis and elucidating the role that sirtuins play in each model will further understanding and therapeutic application of the sirtuins.

My studies of Sirt5 in mice and in vitro have implicated various metabolic pathways (particularly FAO) and the use of various pharmacological modulators of these same pathways is an auspicious future direction for the development of an AKI therapeutic.

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